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Award Number:
W81XWH-06-1-0061

TITLE:
Targeted Elimination of PCDH-PC Expressing Prostate Cancer Cells for Control of Hormone-Resistant Prostate Cancer

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REPORT DATE: November 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

x Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
23-11-2009	Annual	24 OCT 2008 - 23 OCT 2009

4. TITLE AND SUBTITLE
Targeted Elimination of PCDH-PC Expressing Prostate Cancer Cells for Control of Hormone-Resistant Prostate Cancer

5a. CONTRACT NUMBER
W81XWH-06-1-0061

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Ordway Research Institute
150 New Scotland Ave
Albany, NY 12208

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Protocadherin-PC (PCDH-PC or PCDH11Y) is a human-, male-specific gene product that is upregulated in prostate cancer cells by androgen deprivation therapy. Silencing of this gene product with PCDH-PC-specific siRNA drastically induced the death of prostate cancer cells cultured in the absence of androgens and we have proposed that PCDH-PC expression knockout by shRNAs or antisense oligonucleotides (ASOs) might be useful in preventing the development of castration-recurrent prostate cancer in prostate cancer patients. To this end, we have created functional shRNA vectors and ASOs capable of suppressing PCDH-PC expression and we have also created a monoclonal antibody that allows us to detect PCDH-PC protein in cell extracts or tissues. The antibody preferentially recognizes prostate cancer cells in human prostate specimens. Knockdown of PCDH-PC expression by the shRNA vectors is more efficient than with siRNA and is apparently sufficient to kill prostate cancer cells even under conditions where androgen is available and this further supports the idea that PCDH-PC contributes to the biological properties and survival of prostate cancer cells.

15. SUBJECT TERMS
None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE		9	19b. TELEPHONE NUMBER *(include area code)*

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Annual Progress Report

DOD Project: DAAMRDC W81XWH-06-00
Title: *Targeted Elimination of PCDH-PC Expression for Control of Hormone-Resistant Prostate Cancer*
Principal Investigator: Ralph Buttyan, Ph.D.
Progress Period: November 2008 through November 2009

Background: Protocadherin-PC (PCDH-PC) is an androgen-repressed gene encoded on the human Y-chromosome that is over-expressed in prostate cancer cells that were selected for resistance to apoptosis (1). Transfection of this gene product back into prostate cancer cells induces apoptosis- and hormone-resistance (1,2). PCDH-PC is also highly upregulated in androgen-sensitive human prostate cancer cells when they are exposed to androgen-free conditions (1). In our preliminary studies, we found that a small interfering (si) RNA (siRNA) that targets and suppresses the expression of PCDH-PC was able to induce prostate cancer cells under androgen-free conditions. Based upon these results, we proposed that agents that target and block PCDH-PC expression might be used in conjunction with hormonal therapy to increase response and survival of advanced (metastatic) prostate cancer patients. The work in this project will develop and test strategies to suppress PCDH-PC expression in prostate cancer cells based on shRNA and antisense oligonucleotide targeting. Other work will test whether cultured human prostate cancer cells (LNCaP) that are crippled with regards to their ability to upregulate PCDH-PC (by transfection with a PCDH-PC specific targeting shRNA), are killed when they transferred to androgen-deprived medium and whether these cells are unable to form hormone-refractory tumors when xenografted into male immunodeficient mice that are subsequently castrated. We will also test whether the treatment of LNCaP-xenografted mice with antisense oligonucleotides that target PCDH-PC, prevent these mice from developing hormone refractory tumors after they are castrated. In summary, the work in this project will pre-clinically test the idea that PCDH-PC targeting strategies might be a useful adjuvant therapeutic when advanced prostate cancer patients undergo hormonal therapy.

Body: This project has 4 Specific Aims and progress will be discussed for each Aim.

Specific Aim 1**.** Design shRNA expression plasmids that suppress PCDH-PC expression in LNCaP cells and isolate transfected variants of the LNCaP cell line that are unable to induce PCDH-PC expression when cultured in androgen-free medium.

Work Done: We identified two PCDH-PC shRNA vectors (purchased from SBI, Inc., #310598 and 310600) that effectively silence PCDH-PC expression by greater than 90% in cells (293T-T6 or LNCaP-T6) that overexpress this protein (Figure 1). These vectors also carry the gene encoding puromycin resistance so one can use them to create stable cell lines that express the shRNAs. However, when we attempted to stably transfect these shRNA vectors into parental LNCaP prostate cancer cells (that we believed express low levels of PCDH-PC), we were not able to obtain any puromycin-resistant clones. In contrast, transfection by either of two control (non-targeting) shRNA vectors (#30003 and #20003) resulted in abundant puromycin-resistant colonies (> 200/dish). The experiment was repeated twice with similar results. We then considered the possibility that puromycin selection was suboptimal for LNCaP cell lines so we re-cloned the shRNA sequences from 310598 and 310600 (as well as the control non-targeting shRNA sequences) into a different vector

that expressed green fluorescent protein (GFP) as well as the blasticin-resistance protein from separate promoters. Here again, we were easily able to select blasticin-resistant clones when cells were transfected with the control vectors but unable to obtain blasticin-resistant clones from cells transfected with 31058 or 310600. The results suggest that the shRNAs encoded by 31058 and 310600 are generically toxic for LNCaP cells regardless of their androgenic millieu. These results were unexpected since our previous work with LNCaP cells (of high passage) showed that these cells express little, if any PCDH-PC RNA or protein when cultured in normal conditions so we did not anticipate that PCDH-PC targeting shRNA could be lethal for the cells.

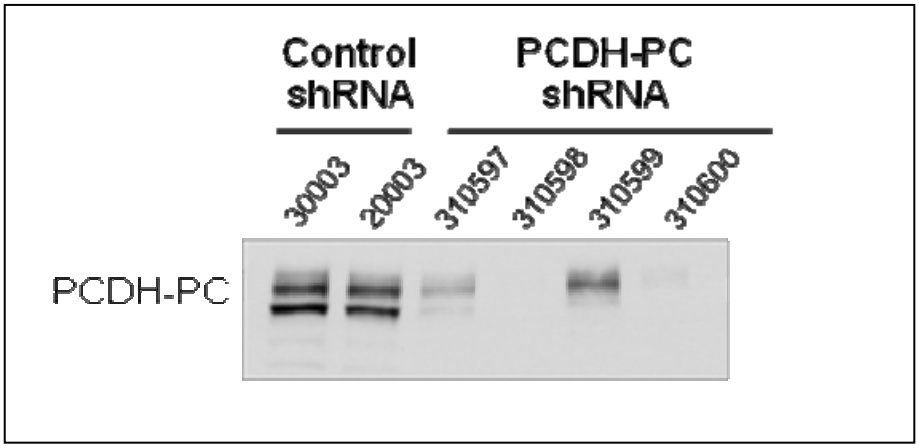
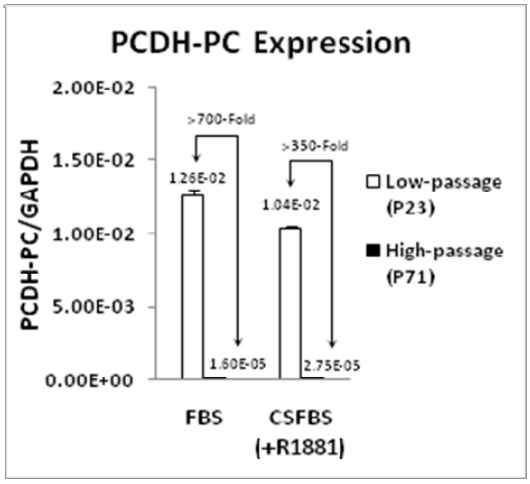


Figure 1 (Above). Reduction of PCDH-PC expression by shRNA targeting vectors. Western blot shows suppression of PCDH-PC expression in 293T cells by PCDH-PC targeting shRNA vectors (#310597-310600) when co-transfected along with a myc-tagged cDNA expression vector containing PCDH-PC cDNA. Cell extracts were prepared 48 hrs after co-transfection and were electrophoresed on an SDS-PAGE gel and blotted onto a filter. The filter was probed with an anti-myc antibody. The levels of myc-tagged PCDH-PC protein in cells co-transfected with PCDH-PC targeting vectors (#310597-310600) were sharply reduced compared to cells co-transfected with the control non-targeting shRNA vectors (#30003 and 20003). However the effects of two of the shRNA targeting vectors (#310598 and 310600) were especially pronounced with virtual complete surpression of PCDH-PC expression in these cells. These results were also reproduced in LNCaP cells.

We then decided to test whether these two PCDH-PC targeting shRNAs were similarly lethal to prostate cancer cells through a mechanism that did not involve PCDH-PC suppression, We transfected control (non-targeting) shRNAs or 310598 and 310600 into PC-3 cells that lack the human Y-chromosome on which the human PCDH-PC gene is encoded. Both control and PCDH-PC-targeting shRNAs gave rise to numerous PC-3 blasticin-resistant colonies so these results continue to support the idea that the parental LNCaP cells that we used must be making some PCDH-PC gene product that is important for their survival.

In hindsight, we recognized that this new work was done with LNCaP cells that were only recently obtained from the ATCC (i.e. low passage cells) in contrast to the high passage variant of these cells which we had previously used for all of our experiments. We then tested, by quantitative PCR (qPCR), whether there was a difference in the amount of PCDH-PC RNA expressed in LNCaP cells as a function of relative passage number. Indeed, (Figure 2a), qPCR quantification of PCDH-PC mRNA levels in low passage vs high passage LNCaP cells showed that the low passage cells express much higher basal levels of PCDH-PC mRNA (> 700-fold) than high passage LNCaP cells when grown under normal (in standard fetal bovine containing medium or in charcoal stripped fetal bovine serum supplemented with androgen) conditions. For our purposes, the results indicate the unexpected results that low passage LNCaP variants already express a considerable amount of PCDH-PC gene product, even in the presence of androgen, (Figure 2a) and the fact that these cells

do not survive transfection with PCDH-PC targeting shRNAs suggests that the cells may be dependent on this expression. It is of interest to note that despite the extensive difference in basal expression of PCDH-PC between low or high passaged LNCaP cells, both cell types further upregulated PCDH-PC mRNA expression to a similar extent (Figure 2b) so our observations of an induction of this gene product in androgen free medium remain supported in either cell type.



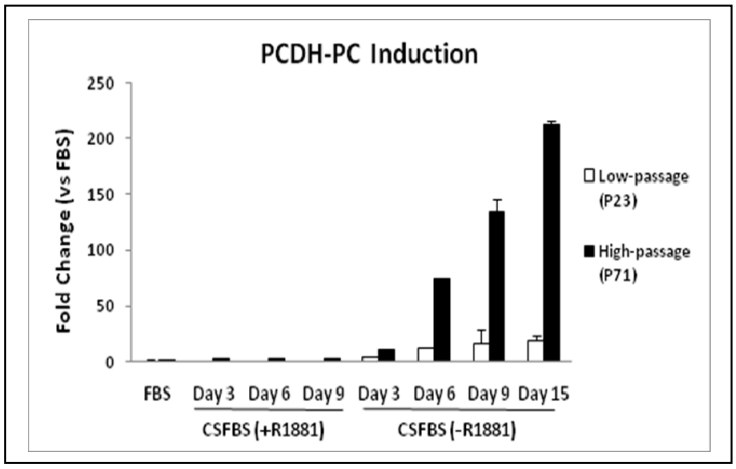


Figure 2 (Above). (A, left panel) Comparative expression of PCDH-PC mRNA between low passage (P23) or high passage (P71) LNCaP cells shows that the low passage cells express more than 700-fold greater levels of PCDH-PC compared to high passage cells in standard Fetal Bovine Serum (FBS) containing medium) and more than 350-fold greater levels when grown in Charcoal-Stripped FBS supplemented with 10 pM R1881 (androgen). (B, left panel) Relative change in expression of PCDH-PC in Low Passage or High Passage LNCaP cells, as indicated, in medium containing androgen (FBS or CSFBS + R1881) or in medium depleted of androgen (CSFBS – R1881). Note that PCDH-PC is induced relatively higher in high passage cells that have low basal expression of PCDH-PC.

Ongoing Work (To Be Completed). Based on this complication, we requested and received a one year no-cost extension on the project so that we can switch back to the use of high passage LNCaP cells for all further work. During the extra year we will produce PCDH-PC shRNA high passage LNCaP cells under this Aim for use in Aim 4.

Specific Aim 2. Design and test antisense oligonucleotides (ASOs) that suppress PCDH-PC expression in prostate cancer cells.

Work Done: We used the sequence of our most successful siRNA (#181, ref 2) to design simple antisense oligonucleotide (19 bases). Shown below in Figure 3 (below), are the results obtained when this ASO was applied to the medium of cells that were previously transfected with a PCDH-PC expression plasmid in which the PCDH-PC protein is “tagged” by a myc-peptide. Expression of the PCDH-PC protein is identified using an anti-myc antibody on Western blots. Results show that this ASO is effective in silencing PCDH-PC expression at an approximately 200 μM concentration or greater. In summary, ASO #181 suitably targets PCDH-PC at concentrations above 200 μM and essentially completes the work described for Specific Aim 2 though we also should consider alternate ASO design based upon other potential targeting sites.

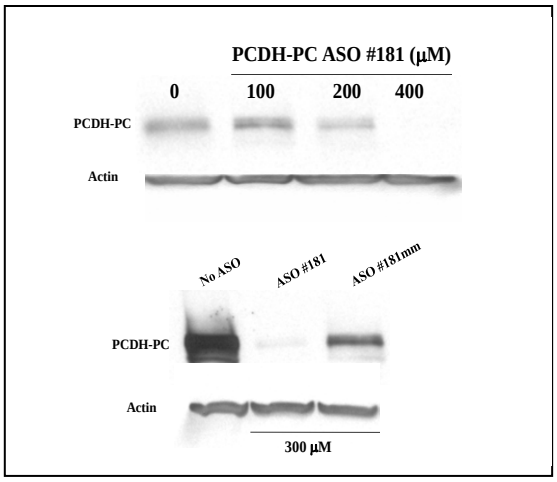


Figure 3. Reduction of PCDH-PC expression by ASO #181. (Top Panel). ASO #181 was transfected into LNCaP cells 24 hrs subsequent to transfection with a myc-tagged PCDH-PC expression vector. 48 hrs later extracts were made of the cells and the extracts were analyzed by Western blot for expression of myc-tagged PCDH-PC protein (110 kd). Results show significant reduction of PCDH-PC expression at 200 uM and higher concentration. (Bottom Panel) The experiment was repeated at a 300 uM ASO concentration and compared to the same concentration of the 181-mm ASO (containing 4 mismatched bases). Results show significant specificity to ASO 181-mediated reduction of PCDH-PC expression.

Specific Aim 3. Test PCDH-PC expression reduction strategies (shRNA or ASO targeting) for ability to induce death of LNCaP cells *in vitro* in androgen-free medium.

Work Done: As discussed in the work under Specific Aim 1, PCDH-PC shRNAs were lethal for low passage LNCaP cells that express high amounts of PCDH-PC, even when these cells were grown in androgen-supplemented medium. During the extension year, we will test this in high passage LNCaP cells that can be induced to express PCDH-PC to high levels when they are deprived of androgen. Additionally, during the last year, we developed an efficient tetracycline-inducible PCDH-PC expression vector (Figure 4, next page) that will enable us to test: 1) whether upregulation of this expression vector confers androgen independent growth in prostate cancer lines other than LNCaP and whether, by blocking its upregulation, we can suppress the acquisition of androgen independent growth.

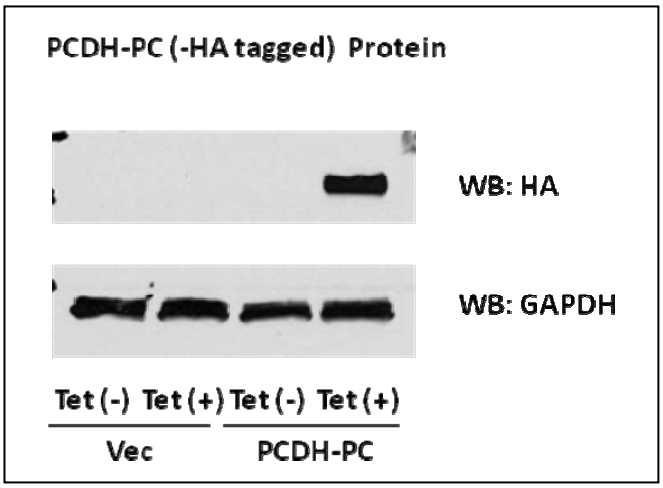


Figure 4. Inducible expression of PCDH-PC (HA-tagged variant) by tetracycline in LNCaP cells. Top shows Western blot using anti-HA antibody to detect HA-tagged PCDH-PC production by stable vector transfected cells (Vec) without (-) or with (+) tetracycline (Left side) or by stable PCDH-PC transfected cells (right side).

Specific Aim 4 Proof-of-principle and pre-clinical testing of targeted PCDH-PC expression knockout combined with androgen deprivation as a strategy for prostate cancer therapy using mouse xenograft tumor models.

No work was done on this Specific Aim during the last period. Although the PI had an approved protocol to conduct this work in the nearby UAlbany Animal Facilities (that extends to Feb 16 2010), the cost of the work for unaffiliated investigators was prohibitive and far outside the budget provided. The Ordway Research Institute (ORI) has now opened its own Animal Facility and the PI has

submitted a protocol for this work that is now under review by the ORI IACUC. This work will be undertaken in the continuation year.

Additional work was also undertaken outside of the original Specific Aims. Though not specified in the original Specific Aims, we feel that this work is relevant to the outcome of this project.

1) *Relationship between the expression of PCDH-PC and expression of Sonic Hedgehog in prostate cancer cells.* PCDH-PC expression is highly upregulated by androgen deprivation. During the last period, we have identified another gene product, Sonic Hedgehog (SHH) that is upregulated coordinately with PCDH-PC in androgen-deprived LNCaP cells. SHH is an embryogenic tissue growth factor and morphogen that mediates signaling through the hedgehog pathway. There is extensive interest in the hedgehog pathway as a mediator of oncogenesis in several human tumor systems including prostate cancer. The graph in Figure 5A shows the relative expression (qPCR data indexed to GAPDH expression in the same specimen) of these two genes as a function of time after androgen withdrawal (CS-FBS). The kinetics of their induction is extremely similar. To further test for a potential relationship between these two gene products, we transfected LNCaP cells with a PCDH-PC expression vector and then compared expression of SHH 48 hrs later to cells that were transfected with a control (empty) vector. As is shown in Figure 4B, SHH expression is increased over 50-fold in the cells that were transfected with PCDH-PC. We are currently testing whether si/shRNA that targets PCDH-PC prevents the upregulation of SHH in androgen deprived medium.

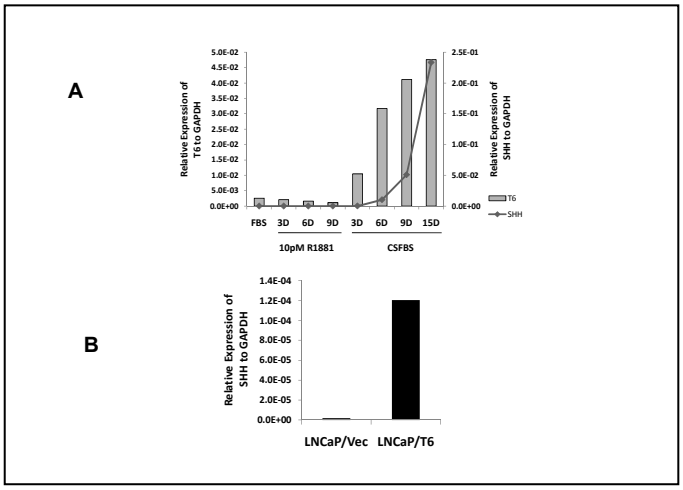


Figure 4. Relationship between expression of PCDH-PC and SHH**. (A)** graph shows relative expression of PCDH-PC (gray bar) or SHH (line) in LNCaP cells grown in androgen-deprived (CS-FBS) medium for up to 15 days. **(B)** Relative expression of SHH in LNCaP cells transfected with empty vector (LNCaP/Vec) or with a PCDH-PC expression vector (LNCaP/T6). Expression of SHH mRNA is increased over 50-fold in PCDH-PC transfected LNCaP cells (48 hrs).

Key Research Accomplishments

- Testing and identification of effective shRNA targeting vectors that suppress expression of PCDH-PC in prostate cancer cells
- Design and testing of an effective anti-sense oligonucleotide that suppresses expression of PCDH-PC in prostate cancer cells
- Demonstration that shRNAs targeting PCDH-PC expression in prostate cancer cells effectively kill these cells even when androgens are present
- Development of highly sensitive monoclonal antibodies that allow detection of native PCDH-PC protein on Western blots
- Preliminary immunostaining of human prostate cancer tissue microarrays indicate that PCDH-PC protein is selectively expressed in prostate cancer cells.
- Identification of a putative relationship between expression of PCDH-PC and SHH.

Reportable Outcomes

Published Manuscripts:

1. Yang, X., Chen, M.-W., Terry, S., Vacherot, F., Bemis, D.L., Capodice, J., Guo, Y. and Buttyan, R. (2006) Complex regulation of human androgen receptor expression by Wnt signaling in prostate cancer cells. *Oncogene*, 25: 3436-3444.
2. Terry, S., Queires, L., Gil-Diez-de-Medina, S., Chen, M.-W., de la Taille, A., Allory, Y., Tran, P.-L., Abbou, C.C., Buttyan, R. and Vacherot, F. (2006) Protocadherin-PC promotes androgen-independent prostate cancer cell growth. *Prostate*, 66: 1100-1113.
3. Chen, M., Tanner, M., Levine, A., Levina, E., Ohouo, P. and Buttyan, R. (2009). Androgenic regulation of hedgehog signaling pathway components in prostate cancer cells. *Cell Cycle*, 8: 149-157.

Biological Resources:

1. shRNA expression vectors (2) that suppress PCDH-PC expression in prostate cancer cells.
2. Antisense Oligonucleotide (#181) that suppresses PCDH-PC expression in prostate cancer cells.
3. Tetracycline-inducible PCDH-PC expression vector to induce PCDH-PC expression in prostate cancer cells by means other than androgen deprivation.
4. Monoclonal antibodies (3) that detect PCDH-PC protein on Western blots.

Conclusions

Work in this project has so far led to the development of biological agents that specifically target and suppress PCDH-PC expression in prostate cancer cells and these agents are useful tools for identifying the role of PCDH-PC in the development of hormone-refractory prostate cancer and potentially for adjuvant therapeutics that might be used, in conjunction with hormone therapy, to improve treatment of advanced prostate cancer. Other work has led to the development of new monoclonal antibodies that recognize PCDH-PC protein and these antibodies might be useful to characterize PCDH-PC expression in human prostate tumor tissues.

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1. Chen, M.-W., Vacherot, F., de la Taille, A., Shen, R., Gil-Diez-de-Medina, S., Chopin, D.K., Friedman, R. and Buttyan, R. (2002) Emergence of protocadherin T6 expression during the acquisition of apoptosis resistance by human prostate cancer cells. *Oncogene*, 21: 7861-7871.
2. Terry, S., Queires, L., Gil-Diez-de-Medina, S., Chen, M.-W., de la Taille, A., Allory, Y., Tran, P.-L., Abbou, C.C., Buttyan, R. and Vacherot, F. (2006) Protocadherin-PC promotes androgen-independent prostate cancer cell growth. *Prostate*, 66: 1100-1113.
3. Yang, X., Chen, M.-W., Terry, S., Vacherot, F., Chopin, D., Bemis, D., Kitajewski, J., Benson, M.C., Guo, Y. and Buttyan, R. (2005) A human- and male-specific protocadherin that acts through the wnt signaling pathway to induce neuroendocrine transdifferentiation of prostate cancer cells. *Cancer Res*. 65: 5263-5271.
4. Chen, M., Tanner, M., Levine, A., Levina, E., Ohouo, P. and Buttyan, R. (2009). Androgenic regulation of hedgehog signaling pathway components in prostate cancer cells. *Cell Cycle*, 8: 149-157.